

## Description

### Novel Potassium Channel Protein

#### Technical Field

The present invention belongs to the field of genetic engineering, and particularly relates to a novel potassium channel protein expressed exclusively in the brain, or an equivalent thereof, a gene encoding the protein or an equivalent thereof, a vector containing the gene, a host cell containing the vector, and so on.

#### Background Art

Potassium channel is a protein which is distributed in the surface membrane of cells and selectively allows potassium ions to pass through it, and it is considered that it takes an important role in controlling membrane potential of cells. Particularly, in nerve and muscle cells, it contributes to the neurotransmission of central and peripheral nerves, pace making of the heart, contraction of muscles and the like by controlling frequency, persistency and the like of action potential. In addition, it has been shown that it is also concerned in the secretion of hormones, adjustment of cell volume, proliferation of cells and the like.

As the classification based on the opening and closing mechanism of the channel, a voltage-dependent potassium channel, an inwardly rectifying potassium channel, a calcium-dependent potassium channel, a receptor coupling type potassium channel and the like have so far been identified. In addition, an ATP-dependent potassium channel, a pH-dependent potassium channel and the like have also been reported. Among them, the voltage-dependent potassium channel has a characteristic in that it opens when the membrane potential is depolarized. In general, potassium ions are present in a non-equilibrium state of about 5 mM outside the cell and about 150 mM inside the cell. Thus, when the voltage-dependent potassium channel is opened by depolarization, potassium ions flow out from intracellular part to extracellular part and, as a result, induce restoration of the membrane potential (repolarization). Accordingly, the opening of voltage-dependent potassium channel induces reduction of excitability of nerve and muscle cells and the like. Also, it causes changes in cellular functions in non-excitatory cells too, such as increase in the driving force for  $\text{Ca}^{2+}$  and subsequent increase in the flow of the same ion into the intracellular part. A compound capable of modifying opening of the voltage-dependent channel has a possibility

of controlling various functions of cells, including excitability of nerve and muscle cells.

Genes of some types of the voltage-dependent potassium channel have been isolated from the brain and heart, and primary structure of the protein has been revealed. Based on the primary structure, it has been suggested that the voltage-dependent potassium channel has six transmembrane domains (S1 to S6) and one ion permeation region (H5). Also, it is assumed that the fourth transmembrane domain S4 contains basic amino acids having positive charge at intervals of 3 to 4 bases and functions as a voltage sensor.

These channels are roughly divided into Shaker type and eag type, based on the similarity of amino acid sequences. The Shaker type is a family having markedly high diversity and can be further divided into four groups of Kv1, Kv2, Kv3 and Kv4. On the other hand, the eag type is constituted by eag, eag-related gene and elk, and it related genes include hyperpolarization activation type potassium channels corresponding to KAT gene cluster and a cation channel which is activated by a cyclic nucleotide.

Regarding the importance of voltage-dependent potassium current in the brain, several findings have been obtained using these cloned voltage-dependent potassium channels. For example, relationship of Kv1.1 with memory

and pain has been suggested by antisense-aided in vivo experiments (Meiri, N. et al. (1997) *Proc. Natl. Acad. Sci. USA*, 94, 4430 - 4434; Galeotti, N. et al. (1997) *J. Pharmacol. Exp. Ther.*, 281, 941 - 949). Regarding Kv3.1, its participation in the excitability of GABA activating interneuron in cerebral cortex has been shown (Massengill, J. et al. (1997) *J. Neurosci.*, 3136 - 3147). On the other hand, some experiments carried out using tetraethylammonium and 4-aminopyridine as non-selective inhibitors of the voltage-dependent potassium channel have also been reported. It has been shown that tetraethylammonium suppresses voltage-dependent potassium current in cerebral cortex nerve cells and also inhibits apoptosis of the same nerve cells (Yu, S.P. et al. (1997), *Science*, 278, 114 - 117). Also, it is known that intraventricular administration of 4-aminopyridine causes epileptic attack. These results suggest a possibility that an agent capable of controlling the activity of voltage-dependent potassium channel in the brain will become a therapeutic agent for central nervous system disorders such as dementia due to disturbance of memory and so on, nerve cell death accompanied by cerebral ischemia, epilepsy and the like.

On the other hand, most of the voltage-dependent potassium channels so far cloned are distributed in a large number of tissues among organs in the whole body. Thus,

even when an agent which acts selectively on a specified voltage-dependent potassium channel is found, there is a possibility that the agent acts on many tissues and thereby induces originally unexpected agent effects. In order to find an agent having less side effects by targeting a potassium channel, it is necessary to clone a potassium channel in which its expressing tissue is restricted.

#### Disclosure of the Invention

An object of the present invention is to provide a novel potassium channel protein expressed exclusively in the brain, as a target of therapeutic agents for central nervous system disorders such as dementia, cerebral ischemic disorder, epilepsy and the like, and another object of the present invention is to provide a method for screening compounds and peptides capable of modifying activity of the same potassium channel protein, which are useful as therapeutic agents for central nervous system disorders, and a novel agent for use in the treatment of central nervous system disorders, which specifically acts upon the central nervous system and generates less side effects.

With the aim of solving the aforementioned problems, the present inventors have conducted intensive studies and, as a result, succeeded in isolating a gene coding for a

novel potassium channel protein expressed exclusively in the brain. The inventors have also succeeded in expressing the novel potassium channel protein expressed exclusively in the brain and establishing a method for the screening of compounds and peptides capable of modifying activity of the same potassium channel protein.

The present invention relates to:

- 1) a potassium channel protein or an equivalent thereof, which has an amino acid sequence selected from either of Sequence Nos. 2 and 6, or an amino acid sequence resulting from said amino acid sequence by substitution, deletion or insertion of certain amino acid(s), and is expressed exclusively in the brain,
- 2) the potassium channel protein or an equivalent thereof according to the item 1), which is expressed exclusively in the human brain,
- 3) a potassium channel protein which has an amino acid sequence selected from either of Sequence Nos. 2 and 6,
- 4) a gene which has a gene sequence encoding the potassium channel protein or an equivalent thereof described in the items 1) to 3),
- 5) a gene which has a gene sequence encoding an amino acid sequence selected from either of Sequence Nos. 2 and 6,
- 6) a gene which has a gene sequence selected from either of the 6th to 3257th gene sequence of Sequence No. 1 or the

4th to 3057th gene sequence of Sequence No. 5, or a gene which is degenerate with respect to said gene,  
7) a vector which contains the gene of the items 4) to 6),  
8) a host cell which contains the vector of the item 7), or  
9) a method for producing the potassium channel protein described in the items 1) to 3), which uses the host cell of the item 8).

The terms to be used in the present invention are explained in the following.

The term "substitution, deletion or insertion of amino acid" means that one or a plurality of amino acids are substituted, deleted or inserted in the amino acid sequence selected from either of the Sequence Nos. 2 and 6.

The term "expressed exclusively in the brain" means that it is expressed in the brain but not expressed in the heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestines, large intestine and peripheral blood leukocytes, illustratively, it means that when Northern blotting is carried out under the conditions of Examples, the signal is detected only in the brain and not detected in the heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestines, large intestine and peripheral blood leukocytes.

Also, the term "equivalent" means a protein having a sequence in which one or a plurality of amino acids are substituted, deleted or inserted in the amino acid sequence of the protein which is expressed in the brain but not expressed in the heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestines, large intestine and peripheral blood leukocytes, but still having the same functions when compared with the protein without alteration of its amino acid sequence.

The term "human origin" means that it is the same amino acid sequence of a potassium channel protein expressed in human.

In this connection, the potassium channel and the potassium channel protein are used as synonyms.

The novel potassium channel protein of the present invention or an equivalent thereof may be any potassium channel protein or an equivalent thereof, with the proviso that it is expressed exclusively in the brain, but is preferably a human origin. Illustratively, a potassium channel protein or an equivalent thereof, which has an amino acid sequence selected from either of Sequence Nos. 2 and 6, or has an amino acid sequence in which one or a plurality of amino acids in the amino acid sequence selected from either of Sequence Nos. 2 and 6 are



substituted, deleted or inserted, and is expressed exclusively in the brain, is included in the present invention and is preferably a human origin. More preferred is a potassium channel protein which has an amino acid sequence selected from either of Sequence Nos. 2 and 6.

The gene which has a gene sequence encoding the novel potassium channel protein of the present invention or an equivalent thereof may be any gene which has a gene sequence encoding a potassium channel protein or an equivalent thereof expressed exclusively in the brain, but is preferably a gene which encodes a potassium channel protein of human origin. Illustratively, a gene which encodes a potassium channel protein or an equivalent thereof having an amino acid sequence selected from either of Sequence Nos. 2 and 6, or a gene which encodes a potassium channel protein or an equivalent thereof having an amino acid sequence in which one or a plurality of amino acids in the amino acid sequence selected from either of Sequence Nos. 2 and 6 are substituted, deleted or inserted, and is expressed exclusively in the brain, is included in the present invention, and the gene is preferably a gene which encodes a human origin potassium channel protein or an equivalent thereof. More preferred is a gene which encodes an amino acid sequence selected from either of Sequence Nos. 2 and 6. Most preferred is a gene which has

a gene sequence selected from either of the 6th to 3257th gene sequence of Sequence No. 1 or the 4th to 3057th gene sequence of Sequence No. 5. Also included in the present invention is a gene which hybridizes with the gene of Sequence No. 1 or 5 under a stringent condition.

Hybridization can be carried out in accordance with a known method (Maniatis, T. et al. (1982): "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY). The term "stringent condition" means a condition that a sample after hybridization is washed twice in 2 x SSC containing 0.1% SDS and then subjected to the following washing step.

This washing step means that the washing (65°C) is carried out in 0.5 x SSC containing 0.1% SDS, preferably in 0.2 x SSC containing 0.1% SDS, most preferably in 0.1 x SSC containing 0.1% SDS. A gene which encodes the novel potassium channel protein of the present invention can be obtained by the following methods.

1) Method for producing novel potassium channel gene

a) First production method

mRNA is extracted from human cells or tissue capable of producing a novel potassium channel protein. Two primers between which the channel protein mRNA or a part of the mRNA region is located are used with the thus extracted mRNA as a template. By carrying out a reverse

transcriptase-polymerase chain reaction (to be referred to as RT-PCR hereinafter), the channel protein cDNA or a part thereof can be obtained. Thereafter, the channel protein can be produced by integrating the thus obtained novel potassium channel cDNA or a part of the same into an appropriate expression vector to carry out its expression in a host cell.

Firstly, from human cells or tissue capable of producing the novel potassium channel protein of the present invention, such as human cerebral cortex, mRNA containing that encoding the protein is extracted by a known method. Guanidine thiocyanate-hot phenol method, guanidine thiocyanate-guanidine hydrochloride method and the like can be exemplified as the extraction method, but guanidine thiocyanate-caesium chloride method can be cited as a preferred method. The cells or tissue capable of producing the protein can be identified, for example, by a western blotting method which uses an antibody specific for the protein.

Purification of the mRNA can be made in accordance with a usual method, for example, the mRNA can be purified by the adsorption and elution using an oligo(dT)-cellulose column. The mRNA can be further fractionated by a sucrose density gradient centrifugation or the like.

Alternatively, a commercially available mRNA extracted sample may be used without carrying out the extraction of the mRNA.

Next, single-stranded cDNA is synthesized by carrying out reverse transcriptase reaction of the thus purified mRNA in the presence of a random primer or an oligo dT primer. This synthesis can be carried out in the usual way. The thus obtained single-stranded cDNA is subjected to PCR using two primers between which a partial region of the gene of interest is located, thereby amplifying the novel potassium channel DNA of interest. The thus obtained DNA is fractionated by an agarose gel electrophoresis or the like. As occasion demands, a DNA fragment of interest can be obtained by carrying out digestion of the DNA with restriction enzymes and subsequent ligation.

#### b) Second production method

In addition to the above production method, the gene of the present invention can also be produced using conventional genetic engineering techniques. Firstly, single-stranded cDNA is synthesized using reverse transcriptase, making use of the mRNA obtained by the aforementioned method as a template, and then double-stranded cDNA is synthesized from the single-stranded cDNA. Examples of this method include S1 nuclease method (Efstratiadis, A. et al. (1976), *Cell*, 7, 279 - 288), Land

method (Land, H. et al. (1981), *Nucleic Acids Res.*, 9, 2251 - 2266), O. Joon Yoo method (Yoo, O.J. et al. (1983), *Proc. Natl. Acad. Sci. USA*, 79, 1049 - 1053) and Okayama-Berg method (Okayama, H. and Berg, P. (1982), *Mol. Cell. Biol.*, 2, 161 - 170).

Next, the recombinant plasmid obtained by the above method is introduced into an *Escherichia coli* strain, such as DH 5 $\alpha$ , to effect its transformation, and then a transformant can be selected making use of tetracycline resistance or ampicillin resistance as a marker. When the host cell is *E. coli*, transformation of the host cell can be carried out, for example, by the method of Hanahan (Hanahan, D. (1983), *J. Mol. Biol.*, 166, 557 - 580), namely a method in which the recombinant DNA is added to competent cells prepared in the presence of CaCl<sub>2</sub> and MgCl<sub>2</sub> or RbCl. In this connection, phage vectors such as a lambda system or the like can also be used as the vector in addition to a plasmid.

Regarding the method for selecting a strain containing DNA which encodes the novel potassium channel protein of interest from the transformants obtained above, various methods such as those shown below can be employed.

(i) A screening method which uses a synthetic oligonucleotide probe

An oligonucleotide which corresponds to the entire portion or a part of the novel potassium channel protein is synthesized (in this case, it may be either a nucleotide sequence taking the codon usage into consideration or a plurality of nucleotide sequences as a combination of possible nucleotide sequences, and in the latter case, their numbers can be reduced by including inosine) and, using this as a probe (labeled with  $^{32}\text{P}$  or  $^{33}\text{P}$ ), hybridized with transformant DNA samples denatured and fixed on a nitrocellulose filter and the resulting positive strains are screened and selected.

(ii) A screening method which uses a probe prepared by polymerase chain reaction

Oligonucleotides of sense primer and antisense primer corresponding to a part of the novel potassium channel protein are synthesized, and a DNA fragment which encodes the entire portion or a part of the novel potassium channel protein of interest is amplified by carrying out polymerase chain reaction (Saiki, R.K. et al. (1988), *Science*, 239, 487 - 491) using these primers in combination. As the template DNA to be used herein, cDNA synthesized by reverse transcription reaction from mRNA of cells capable of producing the novel potassium channel protein, or genomic DNA, can be used. The thus prepared DNA fragment is labeled with  $^{32}\text{P}$  or  $^{33}\text{P}$ , and the clone of interest is

selected by carrying out colony hybridization or plaque hybridization using this fragment as a probe.

(iii) A method in which screening is carried out by producing the novel potassium channel protein in other animal cells

Genes are amplified by culturing the transformants, and transfection of animal cells with the genes is carried out (in this case, either a plasmid which can replicate by itself and contains a transcription promoter region or a plasmid which can be integrated into the chromosome of animal cells may be used), thereby effecting production of proteins encoded by the genes on the cell surface. A strain containing cDNA which encodes the novel potassium channel protein of interest is selected by detecting the protein using an antibody for the novel potassium channel protein, or from the original transformants using the channel activity as a marker.

(iv) A method in which the selection is carried out using channel activity for the novel potassium channel protein as a marker

cDNA is integrated into an expression vector in advance, proteins are produced on the cell surface of transformants, and the strain of interest is selected by detecting desired novel potassium channel protein producing strains using the channel activity as a marker.

(v) A method in which the selection is carried out using an antibody for the novel potassium channel protein cDNA is integrated into an expression vector in advance, proteins are produced on the cell surface of transformants, and the strain of interest is selected by detecting desired novel potassium channel protein producing strains using an antibody for the novel potassium channel protein and a second antibody for the first antibody.

(vi) A method which uses a selective hybridization translation system

cDNA obtained from each transformant is blotted for example on a nitrocellulose filter and hybridized with mRNA prepared from the novel potassium channel protein producing cells, and then the mRNA bonded to the cDNA is dissociated and recovered. The thus recovered mRNA is translated into protein in a protein translation system such as injection into *Xenopus* oocyte or a cell-free system such as a rabbit reticulocyte lysate, wheat germ or the like. A strain of interest is selected by the detection using an antibody for the novel potassium channel protein.

The method for collecting DNA which encodes the novel potassium channel protein from the thus obtained transformant of interest can be carried out in accordance with a known method (Maniatis, T. et al. (1982): "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor



Laboratory, NY). For example, it can be made by separating a fraction corresponding to the plasmid DNA from cells and cutting out the cDNA region from the plasmid DNA.

The method for collecting DNA which encodes the novel potassium channel protein from the thus obtained transformant of interest can be carried out in accordance with a known method (Maniatis, T. et al. (1982): "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY). For example, it can be made by separating a fraction corresponding to the plasmid DNA from cells and cutting out the cDNA region from the plasmid DNA.

c) Third production method

The DNA having a nucleotide sequence which encodes the amino acid sequence selected from either of Sequence Nos. 2 and 6 can also be produced by binding a gene fragment produced by a chemical synthesis method. Each DNA can be synthesized using a DNA synthesizer (e.g., Oligo 1000M DNA Synthesizer (Beckman) or 394 DNA/RNA Synthesizer (Applied Biosystems)).

d) Fourth production method

In order to effect expression of the novel potassium channel protein function expressed exclusively in the brain by the substance obtained by genetic engineering techniques making use of the DNA of the present invention, it is not always necessary to have entire portion of the amino acid

sequence selected from either of Sequence Nos. 2 and 6; for example, when a part of the sequence shows functions of the novel potassium channel protein expressed exclusively in the brain, such an amino acid sequence is also included in the potassium channel protein of the present invention. Also, as is known in the interferon gene and the like, it is considered that genes of eucaryote generally show polymorphism (e.g., see Nishi, T. et al. (1985), *J. Biochem.*, 97, 153 - 159), so that there will be a case in which one or a plurality of amino acids are substituted due to the polymorphism or a case in which the nucleotide sequence is changed but the amino acids are not changed. In consequence, even in the case of a protein in which one or a plurality of amino acid residues are substituted, deleted or inserted at one or a plural positions in the amino acid sequence selected from either of Sequence Nos. 2 and 6, it is possible that it has the channel activity and is expressed exclusively in the brain. In the present invention, such a protein is called an equivalent of the novel potassium channel protein.

All genes which encode such equivalents of the novel potassium channel protein and have equivalent nucleotide sequences are included in the present invention. These various types of DNA of the present invention can also be produced by nucleic acid chemical synthesis in accordance

with a usual method such as phosphite triester method (Hunkapiller, M. et al. (1984), *Nature*, 10, 105 - 111), based on the information on the aforementioned novel potassium channel protein. In this connection, codons for each amino acid are well known and can be optionally selected and determined in the usual way, for example by taking codon usage of each host to be used into consideration (Crantham, R. et al. (1981), *Nucleic Acids Res.*, 9, r43 - r74). In addition, partial modification of codons of these nucleotide sequences can be carried out in accordance with a usual method such as site specific mutagenesis which uses a primer comprised of a synthetic oligonucleotide coding for a desired modification (Mark, D.F. et al. (1984), *Proc. Natl. Acad. Sci. USA*, 81, 5662 - 5666).

Determination of the DNA sequences obtained by the above methods a) to d) can be carried out by, for example, the Maxam-Gilbert chemical modification method (Maxam, A.M. and Gilbert, W. (1980): "Methods in Enzymology" 65, 499 - 559) or the dideoxynucleotide chain termination method which uses M13 (Messing, J. and Vieira, J (1982), *Gene*, 19, 269 - 276).

Also, the vector of the present invention, the host cell of the present invention and the potassium channel

protein of the present invention can be obtained by the following methods.

2) Method for producing recombinant protein of the novel potassium channel

An isolated fragment containing a gene coding for the novel potassium channel protein can transform a host cell of other eucaryote by re-integrating it into an appropriate vector DNA. Also, it is possible to effect expression of the gene in respective host cell, by introducing an appropriate promoter and a sequence related to the gene expression into the vector.

Cells of vertebrates, insects, yeast and the like are included in the eucaryotic host cells, and COS cell as a simian cell (Gluzman, Y. (1981), *Cell*, 23, 175 - 182), a dihydrofolate reductase defective strain of Chinese hamster ovary cell (CHO) (Urlaub, G. and Chasin, L.A. (1980), *Proc. Natl. Acad. Sci. USA*, 77, 4216 - 4220) and the like are frequently used as the vertebral cells, though not limited thereto.

The expression vector which can be used in vertebral cells generally has a promoter positioned at the upstream of the gene to be expressed, an RNA splicing region, a polyadenylation region, a transcription termination sequence and the like, and it may further have a replication origin as occasion demands. Examples of the

expression vector include pSV2dhfr which has SV40 early promoter (Subramani, S. et al. (1981), *Mol. Cell. Biol.*, 1, 854 - 864), though not limited thereto.

When COS cell is used as the host cell, a vector which has the SV40 replication origin, can perform autonomous replication and has a transcription promoter, a transcription termination signal and an RNA splicing region can be used as the expression vector, and its examples include pME18S (Maruyama, K. and Takebe, Y. (1990), *Med. Immunol.*, 20, 27 - 32), pEF-BOS (Mizushima, S. and Nagata, S. (1990), *Nucleic Acids Res.*, 18, 5322), pCDM8 (Seed, B. (1987), *Nature*, 329, 840 - 842) and the like. The expression vector can be incorporated into COS cell, for example, by DEAE-dextran method (Luthman, H. and Magnusson, G. (1983), *Nucleic Acids Res.*, 11, 1295 - 1308), calcium phosphate-DNA co-precipitation method (Graham, F.L. and van der Ed, A.J. (1973), *Virology*, 52, 456 - 457) or electroporation (Neumann, E. et al. (1982), *EMBO J.*, 1, 841 - 845), thereby obtaining a desired transformant cell. Also, when CHO cell is used as the host cell, a transformant cell capable of stably producing the novel potassium channel protein can be obtained by carrying out co-transfection of a expression vector together with a vector capable of expressing neo gene which functions as a G418 resistance marker, such as pRSVneo (Sambrook, J. et

al. (1989): "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY) or pSV2-neo (Southern, P.J. and Berg, P. (1982), *J. Mol. Appl. Genet.*, 1, 327 - 341) or the like, and selecting G418 resistant colonies.

The thus obtained transformant of interest can be cultured in the usual way, and the novel potassium channel protein is produced inside the cells or on the cell surface. Regarding the medium to be used in the culturing, it can be optionally selected from various commonly used types depending on the host cell used, and in the case of the aforementioned COS cell for example, a medium such as RPMI-1640 medium, Dulbecco's modified Eagle's minimum essential medium (DMEM) or the like can be used by supplementing it with serum component such as fetal bovine serum (FBS) or the like as occasion demands.

The novel potassium channel protein thus produced in the cells or on the cell surface of the transformant can be separated and purified therefrom by various known separation techniques making use of the physical properties, chemical properties and the like of the channel protein. Illustrative examples of such techniques, to be carried out after solubilization of the channel protein-containing membrane fraction, include usual treatment with a protein precipitant, ultrafiltration, various liquid chromatography techniques such as molecular sieve

chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, affinity chromatography, high performance liquid chromatography (HPLC) and the like, dialysis and combinations thereof. In this connection, the membrane fraction can be obtained in the usual way. For example, it can be obtained by culturing the cells which expressed the novel potassium channel protein on the cell surface, suspending them in a buffer and then homogenizing and centrifuging them. Also, when the channel protein is solubilized using a solubilizing agent as mild as possible (CHAPS, Triton X-100, digitonin or the like), characteristics of the channel can be maintained after the solubilization.

A method for the screening of compounds and peptides capable of modifying activities of the potassium channel protein is included in the present invention. This screening method includes a means of adding an agent to be tested to the system and measuring the index, in which an index of the modification of potassium channel protein in response to a physiological characteristic of the potassium channel protein is measured making use of the thus constructed potassium channel protein which is expressed exclusively in the brain. The following screening methods can be cited as illustrative examples of this measuring system. Also, examples of the agents to be tested include

compounds or peptides which are conventionally known to have potassium channel ligand activities but their ability to selectively modify activities of the potassium channel protein expressed exclusively in the brain is not clear (e.g., compounds described in JP-A-4-178375; the term "JP-A" as used herein means an "unexamined published Japanese patent application"), various known compounds and peptides registered in chemical files but their potassium channel ligand activities are unknown, compounds obtained by combinatorial chemistry techniques and random peptides prepared by employing a method such as phage display and the like. In addition, culture supernatants of microorganisms and natural components originated from plants and marine organisms are also objects of the screening. Also useful are compounds or peptides obtained by chemically or biologically modifying a compound or peptide selected by the screening method of the present invention.

3) Method for the screening of compounds and peptides capable of modifying activities of the novel potassium channel protein

a) Screening method to which voltage-clamp method is applied

Channel activities of the novel potassium channel protein can be measured by the whole-cell voltage-clamp



method. Cells in which the channel protein is expressed are subjected to membrane potential fixation by the whole-cell voltage-clamp method and whole-cell current is measured. A solution containing 145 mM NaCl, 5.4 mM KCl, 2 mM  $\text{CaCl}_2$  and 0.8 mM  $\text{MgCl}_2$  is used as the extracellular liquid, and a solution containing 155 mM KCl is used as the intracellular liquid (patch electrode solution). Compounds and peptides capable of modifying activities of the novel potassium channel protein can be screened by comparing outward currents generated by a depolarization stimulus, namely shifting of membrane potential from the holding potential (e.g., -70 mV) to the depolarization side (e.g., -80 mV), in the presence and absence of an agent to be tested.

b) Screening method utilizing  $\text{Rb}^+$  ion release

In general, the potassium channel can pass  $\text{Rb}^+$  ion similar to  $\text{K}^+$  ion, so that its channel activity can be measured using radioactive isotope  $^{86}\text{Rb}^+$  release as an index. By carrying out incubation of novel potassium channel protein-expressed cells together with  $^{86}\text{RbCl}$  (e.g., 18 hr at 37°C),  $^{86}\text{Rb}^+$  can be incorporated into the cells. The cells are washed with low  $\text{K}^+$  concentration physiological saline (e.g., 4.5 mM  $\text{K}^+$ ) and then suspended in the same solution. When a high  $\text{K}^+$  concentration solution (e.g., 100 mM in final concentration) is added to

the cell suspension, membrane potential of the cells is depolarized and the potassium channel is activated. Since  $^{86}\text{Rb}^+$  inside the cells is thereby released into the extracellular moiety, the radioactivity in the extracellular liquid can be used as an index of the channel activity. Compounds and peptides capable of modifying activity of the novel potassium channel protein can be screened by comparing radioactivity released into the extracellular moiety when the high  $\text{K}^+$  concentration solution is added, in the presence and absence of an agent to be tested.

c) Screening method which uses voltage-sensitive dye or intracellular  $\text{K}^+$  detecting dye

A voltage-sensitive dye or a intracellular  $\text{K}^+$  detecting dye can optically detect changes in the membrane potential or intracellular  $\text{K}^+$  concentration accompanied by the opening of the potassium channel. As the voltage-sensitive dye, RH155, WW781, Di-4-ANEPPS or derivatives thereof can be used. Also, a chimeric protein prepared by inserting the amino acid sequence of green fluorescent protein into the C-terminal intracellular region of Shaker type voltage-dependent potassium channel can be used for the detection of membrane potential (Siegel, M.S. and Isacoff, E.Y. (1997), *Neuron*, 19, 735 - 741). As the intracellular  $\text{K}^+$  detecting dye,  $\text{K}^+$ -binding benzofuran

isophthalate and the like can be used. Since channel activity of the novel potassium channel can be measured by the use of these dyes, compounds and peptides capable of modifying activity of the novel potassium channel protein can be screened by comparing the changing amounts in the presence and absence of an agent to be tested.

A medicament which contains a compound or peptide capable of significantly modifying the activity of potassium channel protein exclusively expressed in the brain, selected by the aforementioned screening as an active ingredient, is included in the present invention.

The medicament of the present invention is characterized in that it has novel pharmacological action to selectively control activities of potassium channel in the brain, and the use of the medicament is for diseases caused by abnormalities (e.g., acceleration, reduction, and denaturation) of potassium channel activities in the brain or those which express and complicate such abnormalities, wherein their illustrative examples include central nervous system disorders such as dementia, cerebral ischemic disorder, epilepsy and the like.

The pharmaceutical preparation which contains a compound or peptide capable of modifying activity of the potassium channel protein of the present invention, as an active ingredient, can be prepared using carriers, fillers

and other additives generally used in the preparation of medicaments, in response to each type of the active ingredient.

Examples of its administration include oral administration by tablets, pills, capsules, granules, fine granules, powders, oral solutions and the like, and parenteral administration by injections (e.g., intravenous, intramuscular or the like), suppositories, transdermal preparations, transmucosal absorption preparations and the like. Particularly, in the case of peptides which are digested by gastric acid, parenteral administration such as intravenous injection or the like, or lower gastrointestinal delivery administration is desirable.

In the solid composition for use in the oral administration according to the present invention, one or more active substances are mixed with at least one inert diluent such as lactose, mannitol, glucose, microcrystalline cellulose, hydroxypropylcellulose, starch, polyvinyl pyrrolidone or aluminum magnesium silicate. In the usual way, the composition may contain other additives than the inert diluent, such as a lubricant, a disintegrating agent, a stabilizing agent and a solubilizing or solubilization assisting agent. If necessary, tablets or pills may be coated with a sugar coating or a film of a gastric or enteric substance.

The liquid composition for oral administration includes emulsions, solutions, suspensions, syrups and elixirs and contains a generally used inert diluent such as purified water or ethyl alcohol. In addition to the inert diluent, this composition may also contain other additives such as moistening agents, suspending agents, sweeteners, flavors and antiseptics.

The injections for parenteral administration includes aseptic aqueous or non-aqueous solutions, suspensions and emulsions. Examples of the diluent for use in the aqueous solutions and suspensions include distilled water for injection use and physiological saline. Examples of the diluent for use in the non-aqueous solutions and suspensions include propylene glycol, polyethylene glycol, plant oil (e.g., olive oil or the like), alcohols (e.g., ethanol or the like), polysorbate 80 and the like. Such a composition may further contain a moistening agent, an emulsifying agent, a dispersing agent, a stabilizing agent, a solubilizing or solubilization assisting agent, an antiseptic and the like. These compositions are sterilized for example by filtration through a bacteria retaining filter, blending of a germicide or irradiation. Alternatively, they may be used by firstly making into sterile solid compositions and dissolving them in sterile

water or other sterile solvent for injection use prior to their use.

The dose is optionally decided by taking into consideration strength of each active ingredient selected by the aforementioned screening method and symptoms, age, sex and the like of each patient to be administered.

#### Brief Description of the Drawings

Fig. 1 shows amino acid sequences of Sequence No. 2 (upper line) and Sequence No. 6 (lower line) potassium channels.

Fig. 2 shows results of Northern analysis on the novel potassium channel in each human organ. A and B represent results on the potassium channel of Sequence No. 2, and C and D on the potassium channel of Sequence No. 6.

Fig. 3 shows results of Northern analysis on the novel potassium channel in each region of the human brain. A and B represent results on the potassium channel of Sequence No. 2, and C and D on the potassium channel of Sequence No. 6.

In Fig. 4, A and B show results of *in situ* hybridization regarding potassium channel (Sequence No. 2) (DG, the granule cell layer of the dentate gyrus; CA1 and CA3, the pyramidal cell layer of the CA1 and CA3 fields of Ammon's horn; Cx, cerebral cortex). C and D represent

enlarged images of cerebral cortex (arrows indicate signals in typical pyramidal cells). An antisense probe (in A and C) or a sense probe (in B and D) was used in the hybridization. The scale bar indicates 1.5 mm (A and B) or 50  $\mu$ m (B and D).

Fig. 5 shows results of the detection of the channel activity of potassium channel (Sequence No. 2) by depolarization stimulus.

Fig. 6 shows results of the detection of the channel activity of potassium channel (Sequence No. 6) by depolarization stimulus.

Fig. 7 shows tail current of the potassium channel shown in Sequence No. 2.

Fig. 8 shows current response of the potassium channel shown in Sequence No. 6 in high potassium extracellular liquid.

#### Best Mode for Carrying Out the Invention

In order to disclose the present invention more illustratively, examples are described in the following, but the present invention is not limited to the examples. In this connection, unless otherwise noted, they can be carried out in accordance with known methods (Maniatis, T. et al. (1982): "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY).

(Example 1) Isolation of gene encoding the novel potassium channel protein

The full-length cDNA encoding the novel potassium channel protein was obtained by RT-PCR using human brain poly A<sup>+</sup> RNA (Clontech) as a template.

In order to isolate gene of the potassium channel protein shown in Sequence No. 2, 5'-GGAATTCC CTA AGA TGC CGG CCA TGC-3' (Sequence No. 3) was used as a forward primer, and 5'-GCTCTAGAGC ACT CTG AGG TTG GGC CGA AC-3' (Sequence No. 4) as a reverse primer (*Eco*RI site and *Xba*I site are added to respective 5'-termini). RT-PCR was carried out by Hot Start method using Pfu DNA polymerase (Stratagene). After carrying out first thermal denaturation at 96°C (1 minute), a cycle of 96°C (10 seconds)/68°C (30 seconds)/72°C (7 minutes) was repeated 35 times. As a result, a DNA fragment of about 3.3 kbp was amplified. This fragment was digested with *Eco*RI and *Xba*I and then cloned using pME18S plasmid. Since the pME18S plasmid contains SR promoter which shows strong promoter activity in animal cells (Takebe, Y. et al. (1988), *Mol. Cell. Biol.*, 8, 466 - 472), it can be used for expressing recombinant protein in animal cells. This plasmid was received from Dr. Saito at Chiba University (Maruyama, K and Takebe, Y. (1990), *Med. Immunol.*, 20, 27 - 32).



Nucleotide sequence of the thus obtained clone pME-E1 was analyzed by dideoxy terminator method using ABI377 DNA Sequencer (Applied Biosystems). The thus revealed sequence is shown in Sequence No. 1 of Sequence Listing.

This sequence has a 3252-base open reading frame (from the 6th to 3257th of Sequence No. 1). The amino acid sequence (1083 amino acids) deduced from the open leading frame is shown in Sequence No. 2 in the Sequence Listing.

In order to isolate gene of the potassium channel protein shown in Sequence No. 6, 5'-GCC ATG CCG GTC ATG AAG G-3' (Sequence No. 7) was used as a forward primer, and 5'-GCC AGG GTC AGT GGA ATG TG-3' (Sequence No. 8) as a reverse primer. RT-PCR was carried out by Hot Start method using TaKaRa LA Taq (Takara Shuzo). After carrying out first thermal denaturation at 98°C (1 minute), a cycle of 98°C (15 seconds)/68°C (3 minutes) was repeated 35 times and then finally 10 minutes of extension reaction was carried out at 72°C. As a result, a DNA fragment of about 3.1 kbp was amplified. This fragment was cloned using pCR2.1 plasmid (Invitrogen). Nucleotide sequence of the thus obtained clone is shown in Sequence No. 5 of Sequence Listing. In order to express the gene in animal cells, it was subcloned in pME18S plasmid and named pME-E2. The Sequence No. 5 contains an open reading frame of 3,054 bases (from 4th to 3057th positions of Sequence No. 5).

Amino acid sequence deduced from the open reading frame (1,017 amino acids) is shown in Sequence No. 6 of Sequence Listing.

Both of the amino acid sequences of the potassium channels have six hydrophobic regions considered to be transmembrane domains (S1 to S6) which are characteristic of voltage-dependent potassium channel. Also, the S4 domain which is considered to be a voltage sensor has a characteristic in that basic amino acids are continued at an interval of 3 amino acids, and a moderately basic sequence which corresponds to the H5 region is also present between S5 and S6. In addition, both amino acid sequences have high mutual homology. Results of the alignment of both amino acid sequences are shown in Fig. 1. Their homology was 48% with the entire amino acid sequences and 70% with the hydrophobic regions (from 227 position Trp to 508 position Tyr of Sequence No. 2 and from 229 position Trp to 482 position Tyr of Sequence No. 6). In this case, MegAlign program of an analyzing software Lasergene (DNASTAR) was used in the analyses of sequence alignment and homology.

(Example 2) Distribution of novel potassium channel gene expression in human tissues

Distribution of the novel potassium channel gene expression was analyzed by Northern blot hybridization. A cDNA fragment corresponding to the C-terminal intracellular region (from 2105th to 2956th positions of Sequence No. 1 for the potassium channel of Sequence No. 2, or from 2241st to 2898th positions of Sequence No. 5 for the potassium channel of Sequence No. 6) was used as a probe. Hybridization of the probe with a membrane on which poly A<sup>+</sup> RNA (2 µg) of each human organ had been blotted was carried out at 42°C (18 hours) in a solution containing 50% formamide, 5 x SSPE, 10 x Denhardt's solution, 2% SDS and 100 µg/ml of denatured salmon sperm DNA. The membrane was finally washed twice with a solution containing 0.1 x SSC and 0.1% SDS (at 55°C on the potassium channel of Sequence No. 2 and at 60°C on the potassium channel of Sequence No. 6, each for 30 minutes).

When Northern analysis was carried out on various human organs (the heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestines, large intestine and peripheral blood leukocyte), a signal of about 4 kb on the potassium channel of Sequence No. 2 and signals of about 4.4 kb and about 7.5 kb on the potassium channel of Sequence No. 6 were detected, all in the brain alone (Fig. 2). That is, it was found that the mRNA of both of the

novel potassium channels is expressed exclusively in the brain. In this connection, selective expression of the Sequence No. 6 potassium channel mRNA in the brain was confirmed also by RT-PCR analysis.

In addition, the Northern analysis was carried out also on various regions of the human brain (cerebellum, cerebral cortex, medulla, spinal cord, cerebral cortex occipital lobe, cerebral cortex frontal lobe, cerebral cortex temporal lobe, putamen, amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra, subthalamic nucleus and thalamus). It was found that mRNA of the potassium channel shown in Sequence No. 2 is expressed exclusively in the telencephalon including cerebral cortex, caudate nucleus, hippocampus and striatum (putamen and caudate nucleus) (Fig. 3 A and B). On the other hand, it was found that mRNA of the potassium channel shown in Sequence No. 6 is expressed frequently in striped body and cerebral cortex (Fig. 3 C and D). Also, weak expression was found in hippocampus and amygdala. Distributions of both potassium channel genes were overlapped.

(Example 3) Distribution of novel potassium channel expression in neurons of the central nervous system

In order to examine expression of the novel potassium channel gene in neurons of the central nervous system, in

situ hybridization analysis of rat brain sections was carried out. The *in situ* hybridization was carried out in accordance with a report (Okumura, K. et al. (1997), *Oncogene*, 14, 713 - 720) using an antisense RNA probe labeled with digoxigenin. A sense probe was used in the control test. These probes were prepared based on a rat potassium channel gene sequence revealed by the following procedure.

In order to obtain partial sequences of rat potassium channel genes, RT-PCR was carried out using 5'-ACC TTC CTG GAC ACC ATC GC-3' (Sequence No. 11) and 5'-CCA AAC ACC ACC GCG TGC AT-3' (Sequence No. 12) as primers. Both primers correspond to a region of from 14 to 20 positions and a region of from 493 to 499 positions of the amino acid sequence of Sequence No. 2, respectively. As a result of carrying out RT-PCR using poly A<sup>+</sup> RNA isolated from rat brain as a template, fragments of about 1.5 kb and about 1.4 kb respectively corresponding to rat orthologous genes of the potassium channels described in Sequence No. 2 and Sequence No. 6 were obtained. Next, based on the nucleotide sequences revealed from the respective fragments, RACE was carried out on them to reveal complete length sequences. RACE was carried out using Rat Brain Marathon-Ready cDNA (Clontech) in accordance with the manufacturer's instruction. Sequence of the rat

orthologous gene of the potassium channel described in Sequence No. 2 is shown in Sequence No. 9, and sequence of the rat orthologous gene of the potassium channel described in Sequence No. 6 is shown in Sequence No. 10.

An antisense sequence or sense sequence of from 2,683 to 3,204 positions of Sequence No. 9 was used as a probe for the aforementioned rat orthologous gene of the potassium channel described in Sequence No. 2. As a result of the *in situ* hybridization, signals were observed in hippocampus only when the antisense probe was used, and the expression was confirmed in the same region (Fig. 4 A and B). The expression in hippocampus was observed in granule cells of the dentate gyrus, pyramidal cells of the CA1 and CA3 fields of Ammon's horn and the like neurons. Specific signals were also observed in pyramidal cells as neurons of cerebral cortex (Fig. 4 C and D). Thus, it was confirmed that the potassium channel is expressed in the neurons of the central nervous system.

A sequence of from 3,140 to 3,750 positions of Sequence No. 10 was used as a probe for the aforementioned rat orthologous gene of the potassium channel described in Sequence No. 6. It was found that this potassium channel was strongly expressed in neurons of the cerebral cortex.

(Example 4) Induction of the expression of novel potassium channel protein

In order to detect channel activity of the novel potassium channel protein, expression of the protein was induced in an animal cell. As the cell, L929 cell which does not generate current by intrinsic channel through changes in the membrane potential was used. Transfection of the cell was carried out by lipofectAMINE method using pME-E1 plasmid or pME-E2 plasmid.

(Example 5) Detection of channel activity of novel potassium channel protein

The transfected cell was voltage-clamped and whole-cell current was measured using the whole-cell voltage-clamp method. A solution containing 140 mM NaCl, 5.4 mM KCl, 2 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , 15 mM glucose and 10 mM HEPES-Na (pH = 7.4) was used as the extracellular solution, and a solution containing 125 mM KCl, 1 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 11 mM EGTA and 10 mM HEPES-K (pH = 7.2) was used as the intracellular solution.

The cell transfected with pME-E1 was depolarized to voltages between -40 mV and +80 mV at 20 mV intervals for 200 msec from the holding potential of -70 mV (Fig. 5), or the cells transfected with pME-E2 was depolarized to voltages between -60 mV and +60 mV at 20 mV intervals for

200 msec from the holding potential of -120 mV (Fig. 6). As a result, distinctive outward current was induced in both cells. It was found from this result that both of the protein shown in Sequence No. 2 and the protein shown in Sequence No. 6 are voltage-dependent channels.

(Example 6) Selectivity of  $K^+$  ion by novel potassium channel protein

In order to examine selectivity of  $K^+$  ion by the potassium channel shown in Sequence No. 2, tail current was measured. The current was examined by a method similar to Example 5. Based on the tail current (activation of the channel by depolarization stimulus of +80 mV for 200 msec from the holding potential of -70 mV and then repolarization to voltages between -120 mV and -20 mV at 20 mV intervals), the reversal potential in this solution was found to be -80 mV (Fig. 7). Since this value almost coincided with the equilibrium potential of  $K^+$  obtained by the formula of Nernst (-87 mV, 25°C;  $[K]_{out} = 5.4$  mM,  $[K]_{in} = 158$  mM), this channel was considered to have large selectivity for  $K^+$  ion.

An extracellular solution containing 155 mM KCl, 4.5 mM N-methyl-D-glucamine, 2 mM  $CaCl_2$ , 10 mM glucose and 10 mM HEPES (pH = 7.4) was used for the examination of  $K^+$  selectivity of the potassium channel shown in Sequence No.



6. Other conditions were examined by a method similar to Example 5. When depolarization stimulus (between -20 mV and +20 mV at 10 mV intervals for 200 msec from the holding potential of -120 mV) was carried out, the current response was reversed at 0 mV (Fig. 8). It was estimated from this result that the reverse potential was approximately 0 mV. Since this value almost coincided with the equilibrium potential of  $K^+$  obtained by the formula of Nernst (-5 mV, 25°C;  $[K]_{out} = 155$  mM,  $[K]_{in} = 158$  mM), and outward current was induced in Example 5, this channel was considered to have large selectivity for  $K^+$  ion.

#### Industrial Applicability

A novel potassium channel protein expressed exclusively in the brain, a gene encoding this protein, a vector containing this gene, a host cell containing this vector and a method for producing this potassium channel protein were provided by the present invention.

Also, there was provided a method for the screening of new medicaments, particularly new therapeutic agents for central nervous system disorders, in which compounds and peptides capable of modifying activity of the potassium channel protein are screened by allowing the potassium channel protein of the present invention to contact with agents to be tested.

For example, among tissues in which the potassium channel of the present invention shown in Sequence No. 2 was expressed as the result of Example 3, hippocampus is a region where its relationship with memory and learning is strongly suggested (Lavitan, I.B. and Kaczmarek, L.K. (1991), *The Neuron: Cell and Molecular Biology*, Oxford University Press, New York, NY). Particularly, granule cells of the dentate gyrus and pyramidal cells of the CA1 and CA3 fields in which expression of the potassium channel was confirmed form a neural circuit, and various memory inputs are transmitted from the granule cells of the dentate gyrus to pyramidal cells of the CA3 field via pyramidal cells of the CA1 field, mediated by an excitatory synapse which uses glutamic acid as a neurotransmitter. It is considered that long-term changes in the efficiency of synaptic transmission observed in respective synapse, such as long-term increase and long-term repression, are deeply concerned in memory and learning. These long-term changes are controlled by the excitation frequency and excitation strength of nerve cells, and voltage-dependent potassium channels generally have a possibility of being able to control excitability of neurons, so that the potassium channel protein of the present invention which is a voltage-dependent potassium channel has a possibility that

it is concerned in the formation of memory and learning via the excitability control of neurons.

Regarding the medicament which contains a compound or peptide capable of specifically modifying activity of the potassium channel protein of the present invention as its active ingredient, its usefulness is expected for example as a therapeutic agent for central nervous system disorders such as dementia, cerebral ischemic disorders and epilepsy, which acts central nervous system-specifically and has less side effects.